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Investigations toward the selection of fully-modified 4′-thioRNA aptamers: Optimization of in vitro transcription steps in the presence of 4′-thioNTPs

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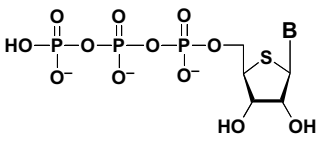
ABSTRACT

We describe herein a method for isolating fully-modified 4′-thioRNA aptamers against human α-thrombin using the SELEX protocol. In order to isolate the desired aptamers, in vitro transcription was examined in the presence of four kinds of 4′-thioribonucleoside triphosphates (4′-thioNTPs) in an effort to afford the fully-modified 4′-thioRNA. The transcription efficiency of the 4′-thioNTPs was first compared with that of the nucleoside 5′-(α-thio)triphosphates (αSNTPs) and found to be less effective than that of the αSNTPs, especially when GTP and/or ATP were substituted for 4′-thioGTP and/or 4′-thioATP. Further attempts to improve its efficiency, including the use of a mutant RNA polymerase instead of the wild type, various additives, and 4′-thioNTP concentrations were unsuccessful. Accordingly, the transcription was performed in the presence of 4′-thioNTPs together with the natural GTP and ATP at the appropriate concentrations. Although this attempt furnished a highly-modified 4′-thioRNA, but not a fully-modified 4′-thioRNA, we eventually succeeded in isolating the fully-modified 4′-thioRNA aptamers by SELEX using optimized transcription conditions, followed by post-modification of the resulting aptamers.

1. Introduction

SELEX (systematic evolution of ligands by exponential enrichment) is a biotechnological method used for isolating nucleic acid ligands,[1] aptamers, from a random pool of single-stranded nucleic acid libraries. Since the resulting aptamers bind to the target molecules, including proteins, with high affinity and specificity in the same manner as monoclonal antibodies, they are expected to become useful biological tools and diagnostic agents.[2,3] In addition, considering that aptamers often inhibit the function of proteins, these molecules should become potential therapeutic agents.[2,4] Recently, Macugen, an aptamer to vascular endothelial growth factor (VEGF), was approved as the first therapeutic aptamer.[5] For the above reasons, the use of SELEX to isolate aptamers is an area currently under intense study.

In order for aptamers to be suitable for versatile applications, they must have higher stability in biological fluids and higher binding affinity. Since natural nucleic acids, especially RNA, is unstable because of its susceptibility to nuclease digestion, stabilization by chemical modification of RNA aptamers would be absolutely required. A highly reliable method of isolating the stabilized RNA aptamers appears to conduct the SELEX protocol, that is, an in vitro transcription step in the presence of chemically modified nucleoside triphosphates (NTPs). Thus far, 2′-modified-2′-deoxynucleoside 5′-triphosphates,[6] nucleoside 5′-(α-*P*-borano)triphosphates,[7] and nucleoside 5′-(α-thio)triphosphates (αSNTPs)[8] are used as modified NTPs for SELEX. Our group has recently reported the successful isolation of modified RNA aptamers using 4′-thiopyrimidine nucleoside triphosphates (4′-thioUTP and 4′-thioCTP; Fig. 1).[9] However, since the substrate susceptibility of NTPs by T7 RNA polymerase is rather restricted, the analogs applicable to SELEX are limited. This being the case, SELEX is generally conducted using two kinds of modified NTPs, such as UTP and CTP analogs, together with natural ATP and GTP for effective transcription by the RNA polymerase. In fact, the aforementioned Macugen was first selected from a modified RNA library transcribed in the presence of 2′-FdUTP and 2′-FdCTP along with the natural ATP and GTP.[10] To confer higher nuclease stability, most of the natural A and G resi-



4′-thioUTP: B = uracil-1-yl
4′-thioCTP: B = cytosin-1-yl
4′-thioATP: B = adenin-9-yl
4′-thioGTP: B = guanin-9-yl

Figure 1. Structure of 4′-thioNTPs.

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dues of the resulting prototype RNA aptamer were post-modified with 2′-OMeA and 2′-OMeG nucleotides.[5] Consequently, achievement of SELEX using four kinds of modified NTPs to afford fully-modified RNA aptamers is considered to be beneficial and challenging.

As described above, our group has succeeded in isolating partially-modified 4′-thioRNA aptamers with potent binding affinity to human α-thrombin using 4′-thioUTP and 4′-thioCTP.[9] We also demonstrated that fully-modified 4′-thioRNA prepared by a chemical approach is 600 times more stable than the natural RNA in human serum.[11] Accordingly, we planned to isolate fully-modified 4′-thioRNA aptamers by SELEX using four kinds of 4′-thioNTPs (4′-thioUTP, 4′-thioCTP, 4′-thioATP, and 4′-thioGTP; Fig. 1). When we began this research project, only one example employing four kinds of αSNTPs to afford phosphorothiolated RNA aptamers was known.[8] Based on these results for isolating chemically modified RNA aptamers, we carried out an investigation into the selection of fully-modified 4′-thioRNA aptamers.

2. Results and discussion

2.1. Screening of reaction conditions to afford 4′-thioRNA transcripts

In order to isolate fully-modified 4′-thioRNA aptamers, a 4′-thioRNA library has to be generated by in vitro transcription in the presence of four kinds of 4′-thioNTPs. The transcription step by T7 RNA polymerase comprises two phases,[12] the *initiation phase* (i.e., starting from +1 to ca. +10 nt in length) and the *elongation phase* (i.e., further elongation to a full-length transcript after the initiation phase). Since the ternary complex consisting of the polymerase, the template DNA, and the resulting RNA is unstable in the initiation phase, formation of abortive transcripts is often observed in this phase, especially when modified NTPs are incorporated.[9,13] Accordingly, successful transcription in the presence of four modified NTPs appears to be difficult, and no example to afford fully-modified transcripts has been reported to date, with one exception. Thus, Ueda et al. examined in vitro transcription by T7 RNA polymerase using αSNTPs and showed that fully-modified phosphorothiolated RNAs could be obtained in 33% yield compared with the reaction in the presence of natural NTPs.[14] As a result of the rather higher transcription efficiency, the isolation of the fully-modified phosphorothiolated RNA aptamers was successfully conducted by Jhaveri et al.[8]

We first examined the transcription in the presence of the 4′-thioNTPs, and compared their efficiencies with those of αSNTPs. In Figure 2, the sequences of the template DNA (template 1) used in this study and the resulting 30mer RNA (RNA 1) were shown. Template 1 was designed for effective incorporation of 4′-thioUTP and 4′-thioCTP since the initial position of the U residue incorporation is +11 and that of the C residue is +13. In fact, a sufficient amount of the transcript (90% yield) was obtained in the presence of 4′-thioUTP, 4′-thioCTP, ATP, and GTP compared with that of the four natural NTPs.[9] Using this template, the in vitro transcription was examined under various conditions. As shown in Figure 3, the transcription in the presence of either 4′-thioCTP (lane 7) or αSCTP (lane 2) afforded the corresponding transcripts in good yield. On the other hand, the transcription using ATP and/or GTP analogs showed a large difference. Thus, contrary to the reactions using αSNTP(s) (lanes 3–6), the efficiencies of 4′-thioNTP(s) (lanes 8–11) were drastically decreased. This result showed that incorporation of 4′-thioNTPs, especially in the initiation phase, was not tolerated for the in vitro transcription by the T7 RNA polymerase, while that of αSNTPs was partially tolerated. Although both 4′-thioNTPs and αSNTPs have a sulfur atom in place of an oxygen atom in their structures, it was revealed that modification of the 4′-oxygen to a sulfur atom had a greater effect on the transcription efficiency. The crystal structure of T7 RNA polymerase has been solved,[15] and its interaction with the template and incoming NTP has been discussed based on the structure of a homologous T7 DNA polymease.[16] According to this report, an interaction of the triphosphate oxygens with the metal (Mg^{2+}) and the RNA polymerase protein (the actual amino acid residues have not been defined) has been suggested. However, such interactions still seem to be operative when the oxygen on phosphorus was substituted to a sulfur atom. On the other hand, little information is reported for

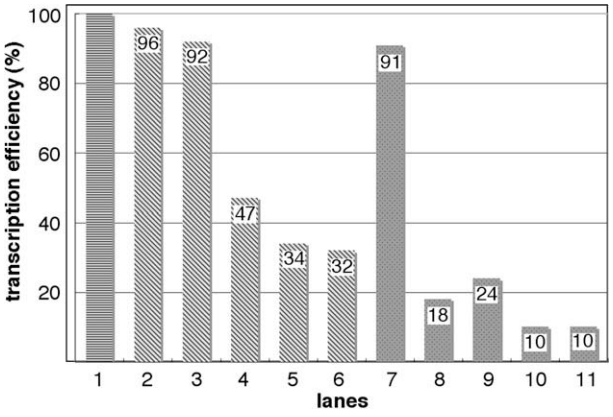

Figure 3. Comparison of transcription efficiency (αSNTPs vs 4′-thioNTPs). In vitro transcriptions were carried out using various NTPs concentrations as described in Section 3; natural NTPs (lane 1); GTP, ATP, UTP plus αSCTP (lane 2) or 4′-thioCTP (lane 7); GTP, CTP, and UTP plus αSATP (lane 3) or 4′-thioATP (lane 8); ATP, CTP, and UTP plus αSGTP (lane 4) or 4′-thioGTP (lane 9); CTP and UTP plus αSGTP and αSATP (lane 5) or 4′-thioGTP and 4′-thioATP (lane 10); UTP plus αSGTP, αSATP, and αSCTP (lane 6) or 4′-thioGTP, 4′-thioATP, and 4′-thioCTP (lane 11). The amount of full-length RNAs was estimated in % based on the conditions in lane 1.

template 1

5′-*TAATACGACTCACTATA*GGGAGAAGAGTACTGTCTATGATCCACCGA-3′
3′-*ATTATGCTGAGTGATAT*CCCTCTTCTCATGACAGATACTAGGTGGCT-5′

RNA 1

5′-pppGGGAGAAGAGUACUGUCUAUGAUCCACCGA-3′
+1 +10 +20 +30

template 2

5′-*TAATACGACTCACTATA*GGGAGAAGGGTATCCGGATCGAAGTTAGTAGGCGGAGTGAGAAGAGGTGACGGTACCAG-3′
3′-*ATTATGCTGAGTGATAT*CCCTCTTCCCATAGGCCTAGCTTCAATCATCCGCCTCACTCTTCTCCACTGCCATGGTC-5′

RNA 2

5′-pppGGGAGAAGGGUAUCCGGAUCGAAGUUAGUAGGCGGAGUGAGAAGAGGUGACGGUACCAG-3′
+1 +10 +20 +30 +40 +50

Figure 2. Sequences of DNA templates and the resulting RNAs by in vitro transcription. The italic regions were T7 promoter sequences.

the interaction with the 4′-oxygen. From our previous investigation, the effective incorporation of 4′-thioNTPs, that is 4′-thioUTP and 4′-thioCTP, was observed in the elongation phase;[9] however this did not take place in the initiation phase. Accordingly, more restricted recognition of the 4′-position of the incoming NTPs appears to occur in this phase.

In order to improve the transcription efficiency, the following attempts were examined: (1) utilization of a mutant T7 RNA polymerase,[17] (2) addition of manganese chloride instead of magnesium chloride,[18] and (3) conducting the reaction at a higher spermidine concentration.[19] However, none of the above efforts improved the transcription efficiency (data not shown). As an alternative, the transcription was conducted at higher modified NTP concentrations. When the concentration of 4′-thioATP was increased to 2.0 mM from the usual 0.4 mM, no alteration of its efficiency was observed (10% vs 11%), while an increase of 4′-thioGTP concentration resulted in the loss of efficiency (10% vs < 1%). Since it is known that GTP, rather than the other NTP substrates, binds very tightly to T7 RNA polymerase in the initiation phase,[20] a modified GTP analog, that is 4′-thioGTP, at a higher concentration, may act as an inhibitor of the transcription. From these results, it was concluded that the in vitro transcription in the presence of four thioNTPs to afford fully-modified 4′-thioRNAs would be difficult.

2.2. Transcription in the presence of GTP and ATP

During the course of our investigation, Burmeister et al. succeeded in isolating fully-modified 2′-OMeRNA aptamers using a mutant T7 RNA polymerase by which NTP analogs modified at their 2′-position are recognized as substrates.[21] However, even in their case, the generation of a fully-modified 2′-OMeRNA library for SELEX was unsuccessful. Accordingly, they carried out in vitro transcription in the presence of 2′-OMeNTPs together with a small amount of the natural GTP. Under these conditions, it was expected that the natural GTP would be preferentially incorporated in the initiation phase, and the 2′-OMeNTPs, including 2′-OMeGTP, would be preferentially used for further elongation. As a result, the authors succeeded in generating a 2′-OMeRNA library (this is not a fully-modified but a highly-modified RNA library), and finally obtained fully-modified 2′-OMeRNA aptamers after optimization.[21] With their successful results as a reference, we examined in vitro transcription by T7 RNA polymerase (wild type). Thus, the reaction was performed in the presence of 4′-thioNTPs together with natural ATP and/or GTP (0.4 mM each as the final NTP concentration), and the transcription efficiencies at various NTP concentrations were determined by PAGE analysis. The results are summarized in Figure 4, in which white and black bars show the results in the presence of four kinds of 4′-thioNTPs together with the natural GTP (column 1) and ATP (column 2), respectively. The gray bars (column 3) indicate the results using both GTP and ATP. The numbers in the parentheses listed along with the lane numbers are the ratios of natural NTP:4′-thioNTP. As can be seen, no full-length transcript was observed in lane 1 (NTP:4′-thioNTP = 0:1). When the ratio of NTP:4′-thioNTP was changed to 1:9, the transcription efficiencies were somewhat increased under all conditions (lane 2). However, no obvious improvement was observed in the presence of either GTP or ATP even if the ratio of NTP was increased (lanes 3–6 of columns 1 and 2). On the other hand, a drastic improvement of the transcription efficiency was observed when both ATP and GTP were included in a ratio of more than 1:1 (lanes 4 and 5 of column 3).

Since the generation of 4′-thioRNA transcripts was successful in the presence of natural GTP and ATP, we next investigated what ratio of 4′-thioGTP and 4′-thioATP against GTP and ATP would allow for competitive incorporation in the resulting transcripts. In order to check the ratio, a longer template DNA (template 2) giving a

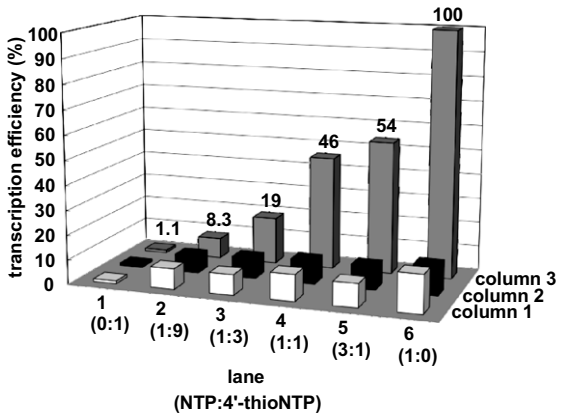

Figure 4. Comparison of transcription efficiency in the presence of four kinds of 4′-thioNTPs together with natural GTP and/or ATP. In vitro transcription was carried out under various NTP concentrations as described in Section 3; 4′-thioNTPs plus GTP (column 1; white bars); 4′-thioNTPs plus ATP (column 2; black bars); 4′-thioNTPs plus GTP and ATP (column 3; gray bars). The ratio in the parentheses is GTP:4′-thioGTP and/or ATP:4′-thioATP. The amount of full-length RNAs was estimated in % based on the conditions in lane 6 of column 3.

59mer transcript (RNA 2) was prepared (Fig. 2). In vitro transcription by T7 RNA polymerase under various NTP concentrations was examined in a similar manner to those in Fig. 3 (when the transcription goes well, the resulting transcript consists of 26 guanine nucleotides and 17 adenine nucleotides among the 59 nucleotides). Then, the resulting full-length transcripts were purified, and hydrolyzed enzymatically, and then, the composition of the nucleosides was analyzed by HPLC. These results are summarized in Figure 5. Thus, the transcription in the presence of 7:1 of GTP:4′-thioGTP and ATP:4′-thioATP afforded 72% of the transcript relative to the conditions in the absence of 4′-thioGTP and 4′-thioATP. Under these conditions, the incorporation ratios were estimated as about 6:1 for GTP:4′-thioGTP and 16:1 for ATP:4′-thioATP from HPLC analysis. As the ratios of 4′-thioGTP and 4′-thioATP were gradually increased, the incorporation ratios of 4′-thioGTP and 4′-thioATP also increased, although the transcription efficiency decreased (conditions 2 and 3). As can be seen from these results, the incorporation of 4′-thioATP versus the natural ATP is rather hard to obtain compared to that of 4′-thioGTP versus the natural GTP. In order to improve the ratio of the 4′-thioATP incorporation, the transcription reaction was conducted at higher 4′-thioATP concentrations[22] (* in Fig. 5, condition 4; 2 mM of 4′-thioATP and the ratio of ATP:4′-thioATP is 1:3). As a result, the incorporation ratio was improved to 2:1 for ATP:4′-thioATP without a drastic loss in transcription efficiency. Throughout these experiments, we were able to generate highly-modified 4′-thioRNA transcripts, and thus started an investigation to isolate the fully-modified 4′-thioRNA aptamers.

2.3. SELEX for the selection of fully-modified 4′-thioRNA aptamers against human α-thrombin

As described above, we showed that in vitro transcription in the presence of four kinds of 4′-thioNTPs, together with appropriate amounts of the natural ATP and GTP, afforded highly-modified 4′-thioRNA transcripts. Accordingly, selection of the 4′-thioRNA aptamers under optimized NTP concentrations (condition 4 in Fig. 5) was next examined. As a target protein for SELEX, human α-thrombin was chosen since we had already isolated its aptamers using 4′-thioUTP and 4′-thioCTP.[9] Single-stranded DNA (ssDNA) templates comprising a 30 nt in randomized positions flanked by constant regions were prepared. After amplification by PCR, the resulting double-stranded DNA (dsDNA) templates were tran-

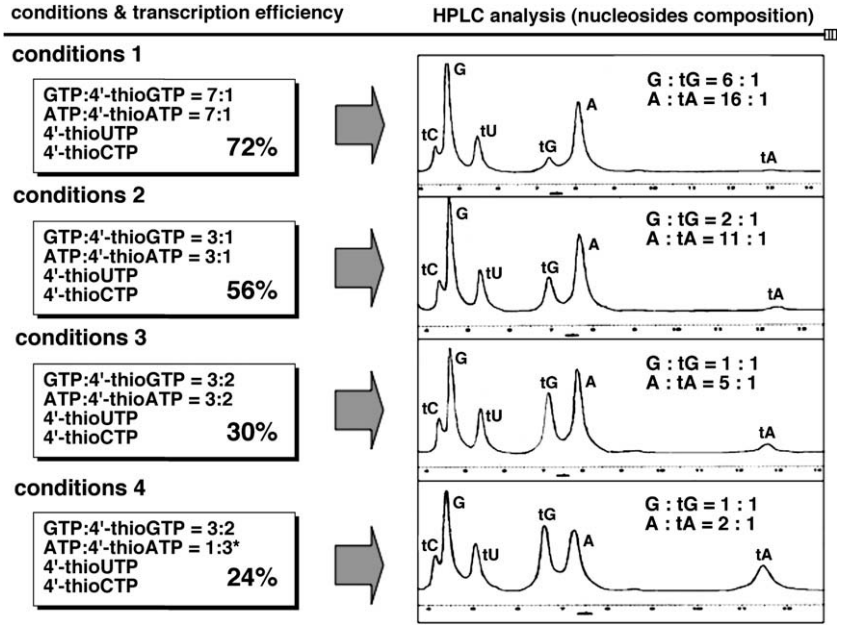

Figure 5. HPLC analysis of nucleosides composition of the resulting transcripts under various NTP concentrations. The NTP concentrations in every condition were presented in Section 3. The amount of full-length RNAs was estimated in % based on the conditions in the presence of GTP and ATP plus 4′-thioUTP and 4′-thioCTP.

scribed by T7 RNA polymerase in the presence of 4′-thioNTPs together with ATP and GTP to afford a 4′-thioRNA library. In general, the transcription by T7 RNA polymerase afforded sufficient amounts of the RNA library throughout the selection. However, in this case, the library obtained at the early stage for the SELEX cycle was too small to subject it to selection even under optimized NTP concentrations. Therefore, the resulting library was labeled at the 5′-end with ^{32}P, and the resulting radio-labeled library was used for selections of up to the fifth round of the SELEX cycle. From the sixth round, the transcription afforded a sufficient amount of the library, and the selection could be done without radio-labeling of the library. In all selections, the natural RNA aptamer against the human α-thrombin (5′-UCCGGAUCGAAGUUAGUAGGCGGA-3′)[23] was mixed with the generated library to increase a selection bias and to isolate more potent aptamers. For the selection processes toward the target protein, a nitrocellulose filter was used, and the counterselections toward the filter were conducted at every round of the SELEX cycle to remove the filter-binding species. The selection processes were repeated under various conditions (see Section 3 for details), and the library from the eighth round was reverse transcribed, amplified, and cloned. Accordingly, three major classes, I, II, and III, emerged from analysis of the 31 clones, in which the most abundant sequences represented 15 of the 31 sequences (Fig. 6). These sequences were not identical with our previous one (i.e., aptamers isolated using 4′-thioUTP and 4′-thioCTP).[9] The predicted sequences, including the constant regions, were prepared by in vitro transcription under the optimal NTP concentrations used for SELEX (i.e., 4′-thioNTPs together with GTP and ATP), and the affinities of the individual full-length transcripts toward human α-thrombin were tested by nitrocellulose filter-binding assay. All sequences showed binding affinities to the target protein. Among them, CI-5 from class I, CII-4 from class II, and CIII were evaluated for their binding affinity. These sequences showed K_d values of 7.2–16.5 nM (Fig. 7), which were similar to that of the aptamer isolated using 4′-thioUTP and 4′-thioCTP.[9] As one might predict, the incorporation of 4′-thioGTP (or 4′-thioATP) competes with GTP (or ATP) for in vitro transcription throughout the SELEX cycles. Therefore, the nucleotide composition of the resulting transcripts, that is the ratio and position of 4′-thioGTP (or 4′-thioATP) incorporation, should change in every transcription step if the sequence is the same. Despite such bias under our SELEX conditions, the fact that the library consists of random sequences that converged on the aptamer sequences shows the viability of this SELEX strategy.

	5'-constant region	random region	3'-constant region	frequency
class I				
CI-1	gggagaagggaaguaacagg	UUUCGUGCCUUUUCU-CGAUGGAGGAGGAGG	gugagaagaggugacgguaccag	(2/31 clones)
CI-2	gggagaagggaaguaacagg	UUUCGUGCCUUUUCUGCGAUGGAGGAGGAGG	gugagaagaggugacgguaccag	(1/31 clones)
CI-3	gggagaagggaaguaacagg	UUUCGUGCCUUUUCU-CGAUGGAGGAGGGGG	gugagaagaggugacgguaccag	(15/31 clones)
CI-4	gggagaagggaaguaacagg	GUUCGUGCCUUUUCU-CGAUGGAGGAGGGGG	gugagaagaggugacgguaccag	(1/31 clones)
CI-5	gggagaagggaaguaacagg	UUUCGUGCCUUUUCU-CGAUGGAG-------	-ugagaagaggugacgguaccag	(1/31 clones)
class II				
CII-1	gggagaagggaaguaacagg	ACAAGCAAUGUACCAAGGGGUG---------	---agaagaggugacgguaccag	(2/31 clones)
CII-2	gggagaagggaaguaacagg	ACAAGCAAUGUACCAAGGGGUGGGAU-GAGG	gugagaagaggugacgguaccag	(1/31 clones)
CII-3	gggagaagggaaguaacagg	ACGAGCAACGUACCAAGGGGUG---------	---agaagaggugacgguaccag	(1/31 clones)
CII-4	gggagaagggaaguaacagg	A-GCAUUAGCUACCAAGGGGUCGGGUUGUGG	gugagaagaggugacgguaccag	(5/31 clones)
CII-5	gggagaagggaaguaacagg	--GCGUAAUGCACCAAGGGGAGGGUUAGUGG	gugagaagaggugacgguaccag	(1/31 clones)
class III				
CIII	gggagaagggaaguaacagg	GUGGGUCUCCGCAUUUGUGCUUCGAU--CCG	gugagaagaggugacgguaccag	(1/31 clones)

Figure 6. Sequences identified in the eighth round library derived from the affinity selection performed on human α-thrombin. The frequency of clones carrying the same sequence is indicated in parenthesis. The sequences common to all ligands (constant regions) are shown in lower case letter and the sequences of the random region in upper case letters.

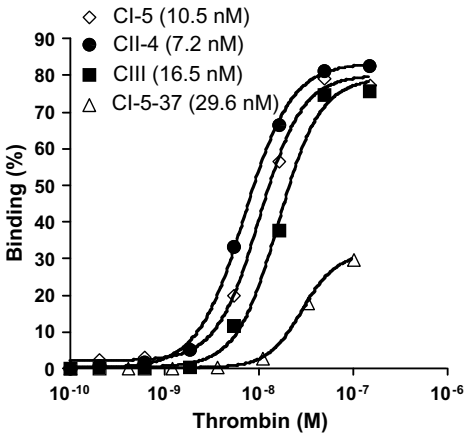

Figure 7. The binding of CI-5, CII-4, CIII, and CI-5-37 (see Fig. 8) to varying concentration of human α-thrombin was determined by nitrocellulose filter partitioning as described in Section 3. The CI-5-37 is a minimized one of CI-5 and was chemically synthesized as a fully-modified 4′-thioRNA.

Since the resulting aptamers include the constant regions along with the random region, minimization of the resulting aptamers is generally performed in order to optimize their sequences. In our investigation, it was also necessary to examine whether the minimized sequences still showed potent binding affinity to the target protein when these were prepared as fully-modified 4′-thioRNA sequences. Therefore, the secondary structure prediction for CI-5, for example, was made using the Zuker RNA mfold computer algorithm[24] to afford a hairpin structure possessing a long stem region. Although it is not clear whether the predicted structure is the actual secondary structure or if it is the best length aptamer, we chemically synthesized a 37mer of fully-modified 4′-thioRNA sequence (CI-5-37) as a candidate of the minimized sequence based on the predicted secondary structure (Fig. 8). As a result, the K_d value of CI-5-37 was estimated as 29.6 nM, although its affinity was slightly lower than the original CI-5 and its maximal binding activity was also decreased (Fig. 7). Since post-modification of the RNA aptamer to the fully-modified 4′-thioRNA sequence resulted in complete loss of its binding affinity in our previous investigation,[9] the fact that the CI-5-37 consisting of a fully-modified 4′-thioRNA showed binding affinity toward human α-thrombin is worth noting. Although further optimization, including structure and length of the resulting aptamer, would likely be required to improve its binding affinity, our results demonstrated that SELEX in the presence of four kinds of thioNTPs together with appropriate amounts of natural GTP and ATP is versatile enough to isolate fully-modified 4′-thioRNA aptamers after optimization. Since 4′-thioRNA showed higher nuclease resistance almost equal to that of 2′-OMeRNA (unpublished results) and thermally stable duplex formation,[11] our results presented here should encourage the development of nuclease-resistant and thermally stable functional RNA molecules by SELEX using commercially available and inexpensive wild type T7 RNA polymerase.

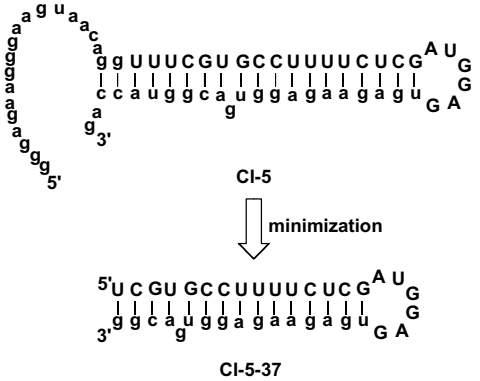

Figure 8. The predicted secondary structure of CI-5 and its minimized sequence (CI-5-37). The upper case letter corresponds to the random region and the lower case letter corresponds to the constant regions.

3. Experimental

3.1. General

Physical data were measured as follows: ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on JEOL-EX270 or JEOL-AL400 instruments in $CDCl_3$, DMSO-d_6, or D_2O as the solvent with tetramethylsilane (^{1}H and ^{13}C) or H_3PO_4 (^{31}P) as an internal standard. Mass spectra were measured on JEOL JMS-D300 spectrometer. UV spectra were measure on Shimadzu UV-2450 spectrophotometer. HPLC was performed on Shimadzu CLASS-LC10 unit using a J'sphere ODN 80 column (4.6 × 150 mm, YMC). TLC was done on Merck Kieselgel F254 precoated plates. Silica gel used for column chromatography was Merck silica gel 5715. T7 RNA polymerase and TaKaRa Ex Taq™ for PCR were purchased from TaKaRa. Supercsript™ II (RNase H^-) Reverse Transcriptase and RNaseOUT™ were from Invitrogen. Nitrocellulose filter (HAWP filter, 0.45 mm) was from Millipore. Human α-thrombin was from Enzyme Research Laboratories. pGEM-T Easy Vector System was from Promega. BigDye Terminator v3.1 Cycle Sequence Kit was from Applied Biosystems. [α-^{32}P]UTP and [γ-^{32}P]GTP were purchased from PerkinElmer. Unlabeled NTPs were from Amersham. Modified NTPs were from TriLink. Radioactive densities of the gel were visualized by a Bio-imaging analyzer (Bas 2500, Fuji). DNA was sequenced using a BigDye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems) on a 377 DNA sequencer (Applied Biosystems). DNA templates for transcription were purchased from SIGMA Genosys and RNA aptamer against α-thrombin was from Hokkaido System Science.

3.2. Synthesis of 4′-thioGTP and 4′-thioATP

Both compounds were prepared starting from appropriately protected 4′-thionucleoside derivatives according to our previous method.[9,25] The synthetic scheme and experimental details were presented in the Supplementary material.

3.3. Comparison of transcription efficiency of 4′-thioNTPs with αSNTPs (investigation shown in Fig. 3)

The in vitro transcription was performed with 40 pmol of template 1 in 20 μL of transcription buffer. The mixture contained either 0.4 mM of natural NTP(s) and modified NTP(s) including [α-^{32}P]UTP (10 μCi), 100 U of T7 RNA polymerase in a 40 mM Tris–HCl buffer (pH 8.0) containing 8 mM $MgCl_2$ and 2 mM spermidine, 40 U of RNaseOUT™, and 5 mM DTT. Reaction mixtures were incubated at 37 °C for 3 h, and 2 μL aliquots of the reaction mixture were added to 4 μL of loading buffer (50 mM EDTA, 10 M urea, 0.1% bromophenol blue, and 0.1% xylene cyanol). The mixtures were then analyzed by electrophoresis on 20% polyacrylamide gel containing 7 M urea. Radioactive densities of the gel were visualized using a Bio-imaging analyzer, and the transcription efficiencies were estimated in % based on the conditions in the presence of natural NTPs.

3.4. In vitro transcription in the presence of 4′-thioNTPs together with GTP and/or ATP (investigation shown in Fig. 4)

The in vitro transcription was performed with 40 pmol of template 1 in 20 μL of transcription buffer. The mixture contained 0.4 mM each of 4′-thioUTP and 4′-thioCTP in every entries, plus

0.4 mM of 4′-thioATP and 0.4 mM of [GTP + 4′-thioGTP] with appropriate ratio (column 1), plus 0.4 mM of [ATP + 4′-thioATP] with appropriate ratio and 0.4 mM of GTP (column 2), or plus 0.4 mM each of [GTP + 4′-thioGTP] and [ATP + 4′-thioATP] with appropriate ratio (column 3). The mixture also included [γ-^{32}P]GTP (10 μCi), 100 U of T7 RNA polymerase in a 40 mM Tris–HCl buffer (pH 8.0) containing 8 mM $MgCl_2$ and 2 mM spermidine, 40 U of RNaseOUT™, and 5 mM DTT. Reaction mixtures were incubated at 37 °C for 3 h, and 2 μL aliquots of the reaction mixture were added to 4 μL of loading buffer (50 mM EDTA, 10 M urea, 0.1% bromophenol blue and 0.1% xylene cyanol). The mixtures were then analyzed by electrophoresis on 20% polyacrylamide gel containing 7 M urea. Radioactive densities of the gel were visualized using a Bio-imaging analyzer, and the transcription efficiencies were estimated in % based on the conditions in the presence of 0.4 mM each of 4′-thioUTP and 4′-thioCTP plus GTP and ATP.

3.5. Analysis of nucleosides composition of the resulting 59mer transcripts (investigation shown in Fig. 5)

In the similar manner as described above, in vitro transcription using template 2 was performed in the presence of 0.4 mM each of 4′-thioUTP and 4′-thioCTP, plus [GTP + 4′-thioGTP] = [0.7 mM + 0.1 mM] and [ATP + 4′-thioATP] = [0.7 mM + 0.1 mM] (condition 1), plus [GTP + 4′-thioGTP] = [0.6 mM + 0.2 mM] and [ATP + 4′-thioATP] = [0.6 mM + 0.2 mM] (condition 2), plus [GTP + 4′-thioGTP] = [0.6 mM + 0.4 mM] and [ATP + 4′-thioATP] = [0.6 mM + 0.4 mM] (condition 3), or plus [GTP + 4′-thioGTP] = [0.6 mM + 0.4 mM], and [ATP + 4′-thioATP] = [0.66 mM + 2.0 mM] (condition 4). For the reaction to estimate the transcription efficiency, [γ-^{32}P]GTP (10 μCi) was also added to the reaction mixture. After being incubated at 37 °C for 3 h, the transcripts were purified on 20% denaturing polyacrylamide gel. The resulting full-length transcripts (0.2 OD) were incubated with snake venom phosphodiesterase (6 mL), RNase A (10 μg), and alkaline phosphatase (0.5 U) in a buffer containing 100 mM Tris–HCl (pH 7.7) and 2 mM $MgCl_2$ (total 516 μL) at 37 °C for 12 h. After the reaction mixture was heated in boiling water for 5 min, the enzymes were removed from reaction mixture by filtration with Micropure®-EZ device (Millipore), and the filtrate was concentrated. Nucleoside composition was determined by analysis of the residue with reverse-phase HPLC, using a J'sphere ODN 80 column (4.6 × 150 mm) with a linear gradient of acetonitrile (from 3.5% to 5% over 30 min) in 0.1 N TEAA buffer (pH 7.0).

3.6. SELEX protocols

SELEX was carried out essentially according to reported methods.[9,23] Single-stranded DNA (ssDNA) templates with a 30 nt variable region (5′-GGGAGAAGGGAAGTAACAGG-N30-GTGAGAAGAGG TGACGGTACCAG-3′; 73mer), a forward primer (5′-GCTCTAGATAA TACGACTCACTATAGGGAGAAGGGAAGTAACAGG-3′; 45mer) and a reverse primer (5′-CTGGTACCGTCACCTCTTCTCAC-3′; 23mer) were prepared. The ssDNA templates were converted to double-stranded DNA (dsDNA) by PCR with the forward and reverse primers. The resulting DNA templates were transcribed using T7 RNA polymerase in the presence of 0.4 mM each of 4′-thioUTP and 4′-thioCTP, plus [GTP + 4′-thioGTP] = [0.6 mM + 0.4 mM] and [ATP + 4′-thioATP] = [0.66 mM + 2.0 mM]. From first to fifth rounds, every transcriptions were performed using T7 RNA polymerase (250 U) in a buffer (100 μL) at 37 °C for 16 h, and the resulting full-length transcripts were purified on 12% denaturing polyacrylamide gel after labeling with ^{32}P at their 5′-end for the selection. From sixth to eighth rounds, the transcriptions were performed using T7 RNA polymerase (2500 U) in a buffer (1000 μL) at 37 °C for 16 h, and the resulting full-length transcripts were used for the selection without 5′-end labeling. The resulting library was denatured in annealing buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$) at 90 °C for 5 min and allowed to cool at 25 °C for 20 min prior to each round of selection. In addition, the library was counterselected by passing through a nitrocellulose filter to remove filter-binding species. For the first round of selection, human α-thrombin (1000 nM) was mixed with radio-labeled 4′-thioRNA library together with unlabeled RNA aptamer (5′-UCCGGAUCGAAGUUAGUAGGCGGA-3′)[23] (5 nM + 500 nM) in the selection buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT). After incubation at 37 °C for 5 min, the mixture was separated by filtration through a nitrocellulose filter to collect thrombin-binding species and washed with 1 mL of the selection buffer. The separated species were eluted from the filter by phenol (400 μL) and freshly prepared 7 M urea (200 μL). Ethanol precipitation with 20 mg of glycogen was carried out and the 4′-thioRNAs recovered were annealed to reverse primer, and reverse transcribed by Superscript™ II (RNase H$^-$) at 42 °C for 50 min to give the cDNAs. Following PCR amplification, the resulting dsDNA templates were transcribed in vitro to give the 4′-thioRNA library for the next round of selection. The concentration of the 4′-thioRNA library together with unlabeled RNA aptamer was fixed to approximately 500 nM in each selection, while that of human α-thrombin in the selection buffer was decreased gradually in successive rounds from 1000 nM to 50 nM. All selection processes were carried out at 37 °C. After eight rounds of selection, the selected RNA pool was reverse transcribed and PCR amplified. The resulting DNAs were cloned and sequenced as described above. The secondary structure predictions for the selected aptamers were made using the Zuker RNA mfold computer algorithm.[24]

3.7. Chemical synthesis of thioRNA aptamer

The predicted thioRNA aptamer, CI-5-37, was synthesized on an Applied Biosystem 3400 DNA synthesizer using four kinds of 4′-thioribonucleoside phosphoramidite units. The synthesis and purification of the aptamer carried out according to our previous methods,[11] and its structure was confirmed by measurement of MALDI-TOF/MASS spectrometry on a Voyager-DE pro. CI-5-37: calculated mass, $C_{351}H_{428}N_{143}O_{227}P_{36}S_{37}$ 12583.4 (M–H); observed mass, 12589.9.

3.8. Filter-binding assay and determination of dissociation constant

The equilibrium dissociation constants (K_ds) for the selected aptamers, CI-5, CII-4, CIII, and CI-5-37, were determined by using a constant amount of 5′-end labeled oligonucleotides (0.2 nM) in the selection buffer with increasing concentrations of human α-thrombin (0.2–200 nM). After incubation at 37 °C for 5 min, the mixture was passed through a nitrocellulose filter, and the filter was immediately washed with selection buffer (200 μL × 3). Radioactivity retained on the filter was quantified by a Bio-imaging analyzer and evaluated by calculating the percentage of the input oligonucleotides retained on each nitrocellulose filter in complexes with protein. The data points were fitted to a Scatchard plot to determine the equilibrium dissociation constant by PRISM.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.09.048.

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